

## DIVISION S-2—SOIL CHEMISTRY

### Phosphorus-31 Nuclear Magnetic Resonance Spectral Assignments of Phosphorus Compounds in Soil NaOH-EDTA Extracts

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#### ABSTRACT

Soil P composition can be conveniently determined in alkaline extracts using solution  $^{31}\text{P}$  nuclear magnetic resonance (NMR) spectroscopy, but spectral assignments are based on fragmentary literature reports of model compounds in various extraction matrices. We report solution  $^{31}\text{P}$  NMR chemical shifts of model P compounds, including inorganic phosphates, orthophosphate monoesters and diesters, phosphonates, and organic polyphosphates, determined in a standardized soil P extractant (0.25 M NaOH and 0.05 M EDTA). Signals from nucleic acids (DNA -0.37 ppm, RNA 0.54 ppm) and phospholipids (phosphatidyl choline 0.78 ppm, phosphatidyl serine 1.57 ppm, phosphatidyl ethanolamine 1.75 ppm) could be differentiated in the orthophosphate diester region, and were identified in a sample of cultured soil bacteria. Inorganic and organic polyphosphates could be differentiated by the presence of a signal at -9 ppm from the  $\alpha$  phosphate of organic polyphosphates. Some orthophosphate diesters, notably RNA and phosphatidyl choline, degraded rapidly to orthophosphate monoesters in NaOH-EDTA although DNA, other phospholipids, and orthophosphate monoesters were more stable. Changes in probe temperature had a marked influence on signal intensities and the relative magnitude of signals from orthophosphate monoesters and inorganic orthophosphate, and we suggest that solution  $^{31}\text{P}$  NMR spectroscopy of soil extracts be performed at 20°C.

SOIL P EXISTS in a multitude of chemical forms, which differ widely in their behavior in the soil environment. Information on soil P composition is a fundamental prerequisite to understanding nutrient and organic matter dynamics in both natural and managed systems. However, such information remains limited. Organic P forms are particularly enigmatic, and a large proportion remains unidentified in most soils (Harrison, 1987). Many such compounds prove difficult to extract chemically, but can nonetheless provide a source of P for plant uptake (e.g., Gahoonia and Nielsen, 1992; Chen et al., 2002).

Detailed information on soil P composition can be obtained by alkaline extraction and solution  $^{31}\text{P}$  NMR spectroscopy. This procedure was first used by Newman and Tate (1980) to investigate P in New Zealand grassland soils, since when it has become the method of choice for determining soil P composition (e.g., Hawkes et al., 1984; Adams and Byrne, 1989; Condon et al., 1990; Bedrock et al., 1994; Guggenberger et al., 1996; Makarov et al., 1997; Mahieu et al., 2000; Amelung et al., 2001).

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Solution  $^{31}\text{P}$  NMR has clear advantages over conventional soil P fractionation schemes, which provide no structural information, while the ability of  $^{31}\text{P}$  NMR to distinguish between similar compounds in complex matrices obviates the need for the lengthy sample clean-up and separation procedures involved in chromatography (Anderson and Malcolm, 1974). The extraction procedure can also be improved using a combination of 0.25 M NaOH and 0.05 M EDTA, which recovers a greater amount and range of soil P compounds than other extractants (Cade-Menun and Preston, 1996). First proposed by Bowman and Moir (1993), NaOH-EDTA extraction is now used to study P in soils (Dai et al., 1996; Robinson et al., 1998; Cade-Menun et al., 2002; Turner et al., 2003), animal manures (Crouse et al., 2000), and aquatic sediments (Sundareshwar et al., 2001).

Identification of compounds by solution  $^{31}\text{P}$  NMR is based on their chemical shift relative to an external  $\text{H}_3\text{PO}_4$  standard. Chemical shift is defined by:

$$\frac{V_s - V_R}{V_R} \times 10^6$$

where  $V_s$  and  $V_R$  are the frequencies of the sample and reference standard, relative to that of the applied magnetic field (Wilson, 1987). Chemical shift values are dimensionless and expressed in parts per million (ppm) with the external standard set to 0 ppm. Chemical shifts depend primarily on the degree of molecular shielding around the P nuclei, but are modified by the surrounding chemical environment. For example, variations in ionic strength, pH, probe temperature, and the presence of paramagnetic ions can all induce subtle changes in chemical shift that complicate signal assignments (Gorenstein, 1984; Crouse et al., 2000; Cade-Menun et al., 2002). Such variations are especially important in the complex spectra of soil extracts, which often involve poorly resolved signals from samples containing small amounts of P. Reference texts containing the chemical shifts of P compounds exist (Gorenstein, 1984; Van Wazer and Ditchfield, 1987), but do not include values measured under the sample conditions encountered in soil or sediment extracts, which include high pH and the presence of often considerable concentrations of paramagnetic ions and salts. As a result, most solution  $^{31}\text{P}$  NMR studies of environmental samples rely on literature reports of the chemical shifts of model compounds added as spikes to extraction solutions. This is unsatisfactory, because such reports are fragmentary (Newman and Tate, 1980; Bedrock et al., 1994), and contain chemical shifts determined in various extract matrices (Adams and Byrne, 1989), or

non-alkaline solutions (Nanny and Minear, 1997; Pant et al., 1999).

In the absence of a comprehensive reference for chemical shift assignments, the principal objective of this study was to determine the chemical shifts of a wide range of P compounds in a soil NaOH-EDTA extract matrix as a reference for future solution  $^{31}\text{P}$  NMR studies of soils and sediments. Secondary objectives were to examine the degradation of P compounds during solution  $^{31}\text{P}$  NMR analysis and investigate the effects of probe temperature on spectral quality.

## MATERIALS AND METHODS

We selected a wide range of P compounds representative of those found in the natural environment, including inorganic phosphates, orthophosphate monoesters, orthophosphate dies-

ters, phosphonates, and organic polyphosphates. Compounds were purchased from Sigma Chemicals (UK), except for bis-methylumbelliferyl phosphate (Glycosynth, UK). These were added as spikes to a soil NaOH-EDTA extract and analyzed by solution  $^{31}\text{P}$  NMR spectroscopy. The soil was a Fladbury clay (USDA Fluvaquents) under permanent lowland grassland near Glastonbury, UK (8.0% total C, 0.2% total P, 68% clay, pH 5.0), which was extracted by shaking 5 g of soil with 100 mL of a solution containing 0.25 M NaOH and 0.05 M EDTA for 16 h at 20°C (Cade-Menun and Preston, 1996). The extract was centrifuged ( $10\,000 \times g$  for 30 min), immediately frozen, and then freeze-dried over several days.

Bacteria were cultured from an upland blanket peat from the Upper Teesdale National Nature Reserve, County Durham, UK (32% total C, 0.6% total P, pH 3.9). Soil suspensions (1 g of soil and 10 mL of deionized water, shaken gently for 5 min) were spread thinly on standard agar plates (containing inorganic

**Table 1.** Solution  $^{31}\text{P}$  NMR chemical shifts of model inorganic P compounds in an alkaline soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.

Compound	Chemical formula	Chemical shift	Line width	Peak area	Orthophosphate shift	Comments
		ppm	Hz	%	ppm	
Inorganic orthophosphate (calcium salt)	$\text{H}_3\text{PO}_4$	5.98 to 6.09				
Pyrophosphate (tetra-sodium salt)	$\text{H}_4\text{O}_7\text{P}_2$	-4.39	17.9	100	6.09	
Polyphosphate (sodium salt)	$\text{H}_n\text{O}_{3n+1}\text{P}_n$	-3.88	7.5	2	6.02	
		-3.94	11.9	18		Doublet ( $J = 15.6$ Hz)
		-4.01	12.9			
		-4.10	5.3	4		Doublet ( $J = 18.6$ Hz)
		-4.18	5.5			
		-4.61	3.2	3		
		-4.63	6.1			
		-18.41	5.6	2		Triplet ( $J = 18.6$ Hz)
		-18.49	6.5			
		-18.57	6.2			
		-18.88	3.6	1		Doublet ( $J = 4.5$ Hz)
		-18.90	5.8			
		-18.94	3.6	1		Doublet ( $J = 4.5$ Hz)
		-18.96	5.1			
		-19.08	9.3	15		Triplet ( $J = 16.4$ Hz) of doublets ( $J = 4.5$ Hz)
		-19.10	7.2			
		-19.15	12.1			
		-19.17	10.7			
		-19.21	5.4			
		-19.24	6.4			
		-19.68	3.6	1		Triplet ( $J = 14.5$ Hz)
		-19.74	4.1			
		-19.80	3.1			
		-20.00	3.5	14		Quadruplet ( $J = 14.1$ Hz)
		-20.05	11.5			
		-20.11	10.2			
		-20.17	5.2			
		-20.36	2.7	7		
		-20.42	9.5			
		-20.47	12.5			
		-20.53	11.4	31		
		-20.56	9.3			
		-21.02	2.4	1		Doublet ( $J = 3.0$ Hz)
		-21.57	3.4			
		-22.53	2.7			
		-22.54	4.0			

P) and incubated for 14 d at 30°C. Bacteria were scraped from the agar and added directly to rehydrated soil extract.

For solution  $^{31}\text{P}$  NMR spectroscopy, freeze-dried soil extract (approximately 500 mg) was redissolved in 5 mL of 1 M NaOH and 0.5 mL of  $\text{D}_2\text{O}$  (for signal lock) and spiked with model compound (1–15 mg) or bacteria (24 mg). Redissolving the freeze-dried extract in 1 M NaOH ensures consistent chemical shifts and optimal spectral resolution by maintaining a solution pH >12. Occasional P compounds were dissolved directly in  $\text{D}_2\text{O}$  and NaOH only (i.e. no soil extract).

Solution  $^{31}\text{P}$  NMR spectra were obtained using a Bruker AMX 600 spectrometer (Bruker, Germany) operating at 243 MHz with a 5-mm probe. We used a  $30^\circ$  pulse width, a total acquisition time of 1.5 s (pulse delay 0.808 s, acquisition time 0.673 s) and broadband proton decoupling (additional spectra were acquired for some samples without proton decoupling). The delay time used here allows sufficient spin-lattice relaxation between scans for P compounds in NaOH–EDTA, confirmed by a recent detailed study of relaxation times for P compounds in various soil extractants (Cade-Menun et al., 2002). Temperature was regulated at 24°C. Between 80 and 400 scans were collected to obtain acceptable signals. Chemical shifts were measured relative to an external standard of 85%  $\text{H}_3\text{PO}_4$  after Lorentzian convolution with a width of 10 Hz. We used the deconvolution process of the Bruker WinNMR program to determine chemical shift, line width, and area of individual signals. Spectra were plotted using a line broadening of 5 Hz. Spectra were collected immediately after preparation (usually within 1 h) and repeated at intervals during storage at room temperature to determine degradation, with the same number of scans collected each time. No degradation was observed unless specified.

The effect of probe temperature on solution  $^{31}\text{P}$  NMR spectra was investigated by analyzing (i) a synthetic mixture of model P compounds, and (ii) the soil NaOH–EDTA extract. Probe temperature was varied between 19 and 44°C for the synthetic mixture and between 18 and 37°C for the soil extract. Spectra were recorded for 128 scans (synthetic mixture) or 1024 scans (soil extract), with other machine parameters as described above.

## RESULTS

### Chemical Shifts and Degradation of Model P Compounds in NaOH–EDTA

The chemical shift of inorganic orthophosphate (as  $\text{PO}_4^{3-}$  in the alkaline solution analyzed here) was between 5.98 and 6.09 ppm and remained consistent in the presence of all model compounds and soil bacteria (Table 1, Fig. 1). This chemical shift varies from that of the external  $\text{H}_3\text{PO}_4$  standard (0 ppm) because of the difference in solution pH. The orthophosphate spectra also shows signals from the original soil extract at approximately 0, –4, and between 4 and 6 ppm (Fig. 1). These signals are not apparent in all spectra, depending on the amount of added model P compound.

Inorganic polyphosphate (containing P–O–P bonds) exhibited a complex series of signals corresponding to the positions of the P atoms in the polyphosphate chains (Table 1, Fig. 1). Major groups of signals appeared at –4.0 ppm (terminal P groups), –19.2, –20.1, and –20.5 ppm (penultimate and mid-chain P groups) (Hupfer and Gächter, 1995). Pyrophosphate, a specific inorganic polyphosphate with chain length  $n = 2$ , gave a signal at –4.39 ppm. Inorganic polyphosphates were stable during at least 9 d storage in NaOH–EDTA.

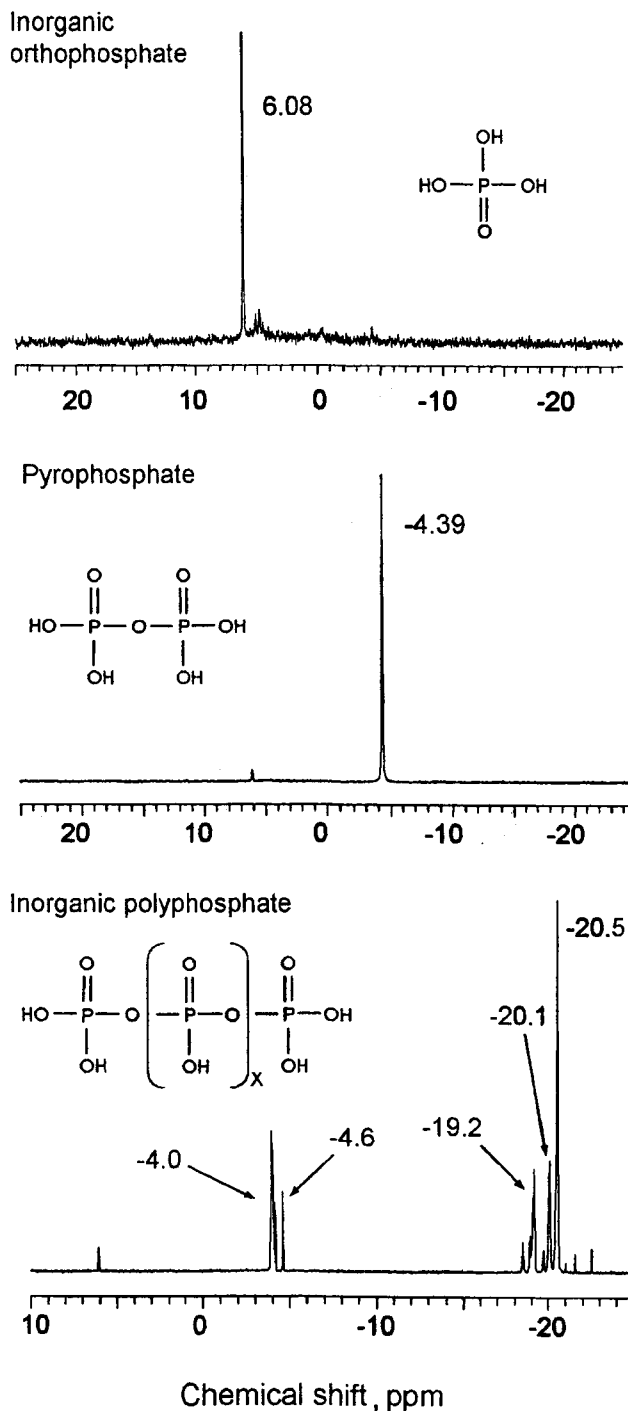
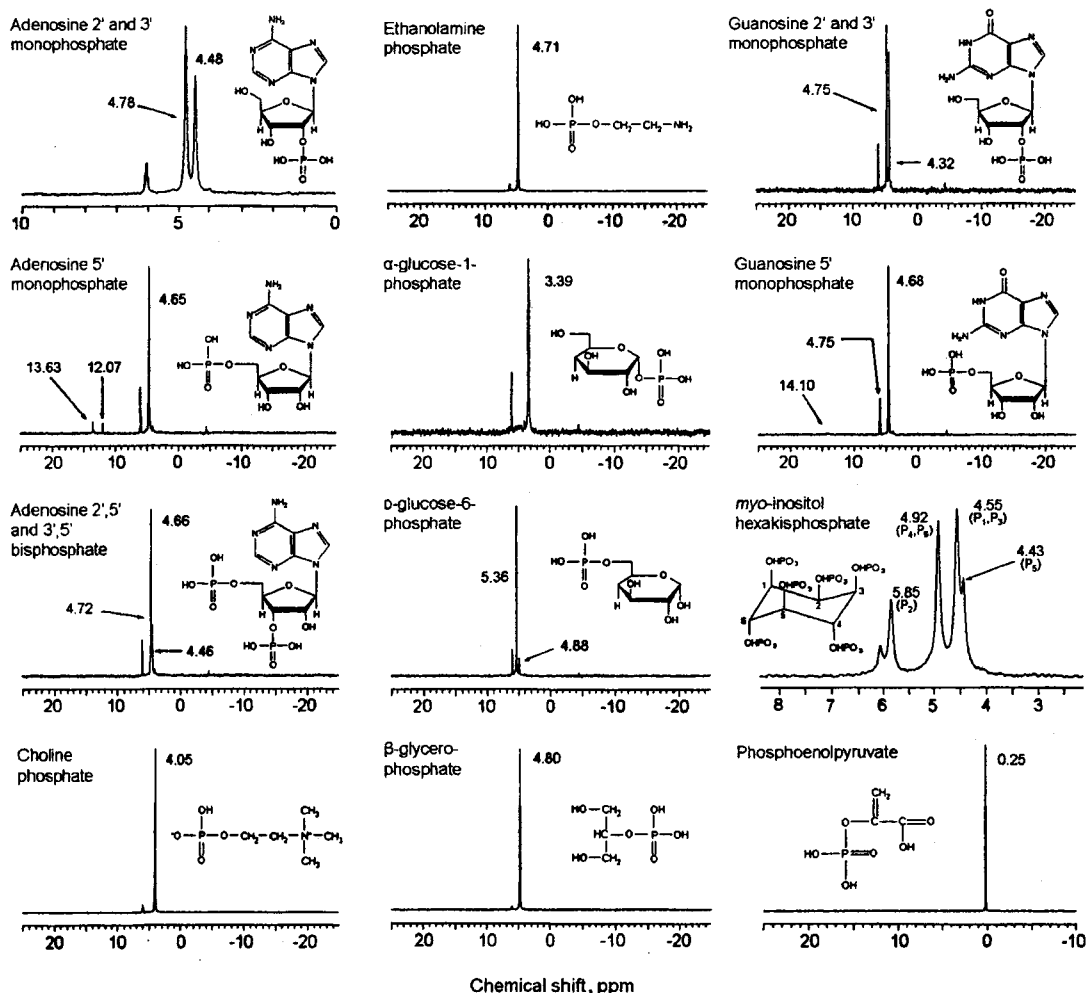


Fig. 1. Solution  $^{31}\text{P}$  NMR spectra of model inorganic P compounds added to a soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.

Orthophosphate monoesters gave signals between 3 and 6 ppm (Table 2, Fig. 2), and did not degrade in NaOH–EDTA. *Myo*-inositol hexakisphosphate (phytic acid) gave four characteristic signals in the ratio 1:2:2:1 at 5.85, 4.92, 4.55, and 4.43 ppm, corresponding to the positions of the P atoms on the inositol ring (Costello et al., 1976; Fig. 2). Other orthophosphate monoesters resonated in this region, including sugar phosphates

**Table 2. Solution  $^{31}\text{P}$  NMR chemical shifts of model orthophosphate monoesters in an alkaline soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.**

Compound	Chemical formula	Chemical shifts	Line width	Peak area	Orthophosphate shift
		ppm	Hz	%	ppm
Adenosine 2' and 3' monophosphate (mixed isomers)	$\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_7\text{P}$	4.48 4.78	17.5 17.2	41 59	6.05
Adenosine 5' monophosphate	$\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_7\text{P}$	4.65 12.07 13.63	19.2 10.1 21.5	91 3 6	6.03
Adenosine 2',5' and 3',5' bisphosphate (mixed isomers)	$\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_{10}\text{P}_2$	4.46 4.66 4.72	14.2 13.6 12.0	16 53 30	6.05
Choline phosphate (phosphorylcholine)	$\text{C}_5\text{H}_{14}\text{NO}_4\text{P}$	4.05	13.4	100	6.03
Ethanolamine phosphate (o-phosphoryl-ethanolamine)	$\text{C}_2\text{H}_7\text{NO}_4\text{P}$	4.71	13.9	100	6.01
$\alpha$ -D-glucose-1-phosphate	$\text{C}_6\text{H}_{13}\text{O}_9\text{P}$	3.39	36.5	100	6.05
D-glucose-6-phosphate	$\text{C}_6\text{H}_{13}\text{O}_9\text{P}$	4.88 5.36	16.8 16.1	9 91	6.02
$\beta$ -glycerophosphate	$\text{C}_3\text{H}_7\text{O}_6\text{P}$	4.80	14.4	100	6.09
Guanosine 2' and 3' monophosphate (mixed isomers)	$\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_7\text{P}$	4.32 4.75	13.6 13.6	45 55	6.05
Guanosine 5' monophosphate	$\text{C}_{10}\text{H}_{14}\text{N}_5\text{O}_7\text{P}$	4.68 4.75 14.10	11.7 9.8 88.3	86 9 5	6.03
<i>myo</i> -inositol hexakisphosphate (phytic acid)	$\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$	4.43 4.55 4.92	21.6 22.9 21.2	16 36 31	6.05
Phosphoenolpyruvate†	$\text{C}_3\text{H}_3\text{O}_6\text{P}$	5.85 0.25	22.7 7.7	17 100	

† Analyzed in  $\text{D}_2\text{O}$  and 1 M NaOH only (no soil extract).**Fig. 2. Solution  $^{31}\text{P}$  NMR spectra of model orthophosphate monoesters added to a soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.**

**Table 3.** Solution  $^{31}\text{P}$  NMR chemical shifts of model orthophosphate diesters in an alkaline soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in 1 M NaOH and  $\text{D}_2\text{O}$ . Results are for freshly prepared compounds unless stated.

Compound	Chemical formula	Time after preparation	Chemical shift	Line width	Peak area	Orthophosphate shift
			ppm	Hz	%	ppm
Adenosine 3',5' cyclic mono-phosphate	$\text{C}_{10}\text{H}_{12}\text{N}_5\text{O}_6\text{P}$		-0.09	15.2	100	6.05
R-(-)-1,1'-binaphthyl-2,2'-diyl hydrogen phosphate†	$\text{C}_{20}\text{H}_{12}\text{O}_4\text{P}$	3 h	7.36	6.2	85	
			5.98	6.1	10	
			0.29	5.1	5	
		24 h	7.36	6.1	67	
			5.98	9.9	13	
			0.29	7.5	20	
		40 d	7.36	6.8	19	
			5.99	5.8	3	
			0.29	8.6	78	
Deoxyribonucleic acid (DNA) (from salmon testes)			-0.37	36.8	72	6.01
			-0.63	45.1	28	
DNA (from herring sperm)			-0.52	36.4	7	
			-0.25	48.4	56	
			0.01	35.2	5	
			0.35	29.5	4	
			0.52	11.6	1	
			4.04	20.4	5	
			4.57	28.2	18	
			4.70	35.2	2	
			4.83	13.7	1	
			5.22	10.9	1	
$\text{N}^6$ , 2'-O-dibutyryl adenosine 3'5'-cyclic monophosphate (bucladesine)	$\text{C}_{18}\text{H}_{24}\text{N}_5\text{O}_8\text{P}$		-0.07	15.5	100	6.05
L- $\alpha$ phosphatidyl choline (lecithin, from fresh egg yolk)	$\text{C}_{10}\text{H}_{19}\text{NO}_8\text{P}(2\text{R})^\dagger$	30 min	5.98	10.9	17	5.98
			0.78	8.1	83	
		2 h	19.60	22.9	5	
			19.45	7.2	4	
			5.99	7.0	6	
			5.50	8.5	2	
			5.15	6.3	4	
			4.82	5.8	7	
			0.78	7.6	65	
			-1.07	33.3	7	
		4 h	19.44	6.1	5	
			5.98	7.5	6	
			5.15	6.8	12	
			4.81	7.0	22	
			0.78	7.6	53	
			0.02	10.4	3	
		24 h	5.98	9.3	7	
			5.15	7.7	34	
			4.81	7.3	59	
		19 d	5.98	11.3	4	
			5.15	9.1	35	
			4.81	9.0	61	
L- $\alpha$ phosphatidyl ethanolamine (cephalin, from <i>Escherichia coli</i> )	$\text{C}_8\text{H}_{12}\text{NO}_8\text{P}(2\text{R})^\dagger$		1.75	97.2	100	6.00
L- $\alpha$ -phosphatidyl-L-serine (from bovine brain)‡	$\text{C}_8\text{H}_{12}\text{NO}_{10}\text{P}(2\text{R})^\dagger$	30 min	5.15	4.8	1	
			1.57	8.4	99	
		4 h	5.15	8.5	3	
			4.82	8.0	2	
			1.57	9.9	95	
		24 h	5.15	8.0	6	
			4.81	10.5	6	
			1.57	8.5	88	
		19 d	5.15	10.0	28	
			4.82	9.5	34	
			1.57	9.3	38	

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(glucose phosphates at 3.39 and 5.36 ppm), mononucleotides (4.32 to 4.78 ppm) and the monoester breakdown products of phospholipids (choline phosphate at 4.05 ppm, ethanolamine phosphate at 4.71 ppm). The only orthophosphate monoester signals not in the 3 to 6 ppm region were aromatic monoesters (see below) and phosphoenolpyruvate (0.25 ppm), a common compound in plant material. The samples of adenosine and guanosine 5' monophosphate also gave small signals between 12 and 14 ppm, which may indicate contaminant aromatic

phosphonic acid esters (see below). Lower inositol phosphate esters (inositol mono- to pentakisphosphates) and isomeric forms other than *myo* (*scyllo*, *D-chiro*, *neo*) also occur in soils and sediments (Turner et al., 2002b), but were not analyzed because of their expense. However, the lower inositol phosphate esters almost certainly give different signals to those of *myo*-inositol hexakisphosphate (Kemmer et al., 1999).

Orthophosphate diesters gave signals between -1.0 and 2.0 ppm (Table 3, Fig. 3). The main signal from

Table 3. Continued.

Compound	Chemical formula	Time after preparation	Chemical shift	Line width	Peak area	Orthophosphate shift
			ppm	Hz	%	ppm
Ribonucleic acid (RNA) Type VI		30 min	0.20	37.7	1	6.01
			0.36	16.8	2	
			0.45	11.2	1	
			0.49	15.7	2	
			0.56	24.4	5	
			0.68	24.0	3	
			0.74	16.0	2	
			0.79	12.0	2	
			0.89	16.4	1	
			4.28	9.9	8	
			4.37	10.3	5	
			4.45	11.0	11	
			4.48	8.4	4	
			4.53	21.2	6	
			4.62	26.3	8	
			4.71	15.5	29	
			4.75	8.9	11	
		24 h	4.28	9.9	12	6.01
			4.37	10.3	9	
			4.45	11.0	13	
			4.48	8.4	6	
			4.53	21.2	1	
			4.62	26.3	1	
			4.71	15.5	36	
			4.75	8.9	20	

† R represents hydrophobic fatty acyl chains, which may not be identical.

‡ Analyzed in D<sub>2</sub>O and 1 M NaOH only (no soil extract).

DNA was at  $-0.37$  ppm, with a shoulder at  $-0.63$  ppm. DNA was stable in NaOH-EDTA and was well separated from RNA at  $0.54$  ppm. However, RNA degraded completely to orthophosphate monoesters within 24 h in the NaOH-EDTA extract. A sample of degraded DNA containing mixed polynucleotides gave similar signals to intact DNA, plus a group of poorly resolved signals between 0 and 1 ppm. Signals in the 4 to 5 ppm region of this spectrum represent mononucleotide degradation products of DNA.

Phospholipids gave signals at  $0.78$  ppm (phosphatidyl choline),  $1.57$  ppm (phosphatidyl serine), and  $1.75$  ppm (phosphatidyl ethanolamine). Of these, phosphatidyl choline (from plants) and phosphatidyl ethanolamine (from microbes) are the most common in soils (Kowalenko and McKercher, 1971). Phosphatidyl choline degraded almost completely within 24 h to orthophosphate monoesters at  $4.81$  and  $5.15$  ppm. In contrast, phosphatidyl serine degraded much more slowly (12% degradation in 24 h), while phosphatidyl ethanolamine showed no degradation in 24 h. The aromatic orthophosphate diester binaphthyl phosphate ( $7.4$  ppm) degraded slowly to a compound at  $0.29$  ppm.

Phosphonates, which contain a C-P bond, gave signals between 12 and 23 ppm (Table 4, Fig. 4). Aminoethyl phosphonates gave signals around 20 ppm (Newman and Tate, 1980), while aromatic phosphonic acid esters (which contain both a phosphonate and an ester bond) appeared between 12 and 14 ppm. Phosphocreatine, which contains a P-N bond rather than a P-C bond as in the phosphonates, gave a signal at  $0.29$  ppm. No degradation of aminoethyl phosphonic acids was detected during several months storage in NaOH-EDTA, but a compound with a chemical shift of  $12.07$  ppm appeared after 24 h in the  $\beta$ -naphthyl phenyl phospho-

nate solution, and increased to the height of the signal from the original compound after storage for 15 d, despite no change in the height of the original signal. Similar weak signals were detected between 12 and 13 ppm in the sample of adenosine 5' monophosphate (Table 2). When a spectrum of 2-aminoethyl phosphonic acid was acquired without proton decoupling, the signal split into seven smaller signals (Fig. 4).

Organic polyphosphates gave multiple signals in the negative region of the spectra (Table 5, Fig. 5). Thus, ADP displayed signals at  $-4.71$  and  $-9.20$  ppm, corresponding to the  $\beta$  and  $\alpha$  phosphates, respectively. Similarly, ATP gave signals at  $-4.28$ ,  $-9.68$ , and  $-19.68$  ppm, corresponding to the  $\gamma$ ,  $\alpha$ , and  $\beta$  phosphates, respectively.

Aromatic orthophosphate esters are routinely used to study the activity of soil and plant phosphatase enzymes (Tabatabai, 1994; Turner et al., 2001a). Colorimetric compounds contain phosphate ester linkages to *para*-nitrophenol, while fluorimetric compounds contain phosphate ester linkages to methylumbelliferyl. The aromatic orthophosphate monoesters analyzed here gave signals at  $0.40$  and  $1.18$  ppm, while the aromatic orthophosphate diesters appeared at  $-7.19$  and  $-10.63$  ppm (Table 6, Fig. 6). The signal from *para*-nitrophenyl phosphate was consistent with a literature report of aromatic orthophosphate monoester signals at  $0.3$  ppm (Bedrock et al., 1994). A signal at  $0.36$  ppm in the spectrum of bis-*para*-nitrophenyl phosphate corresponded to its monoester degradation product *para*-nitrophenyl phosphate.

### Degradation of Bacterial Phosphorus Compounds in NaOH-EDTA

Bacterial organic P compounds degraded rapidly in alkaline solution (Table 7, Fig. 7). In the fresh sample,

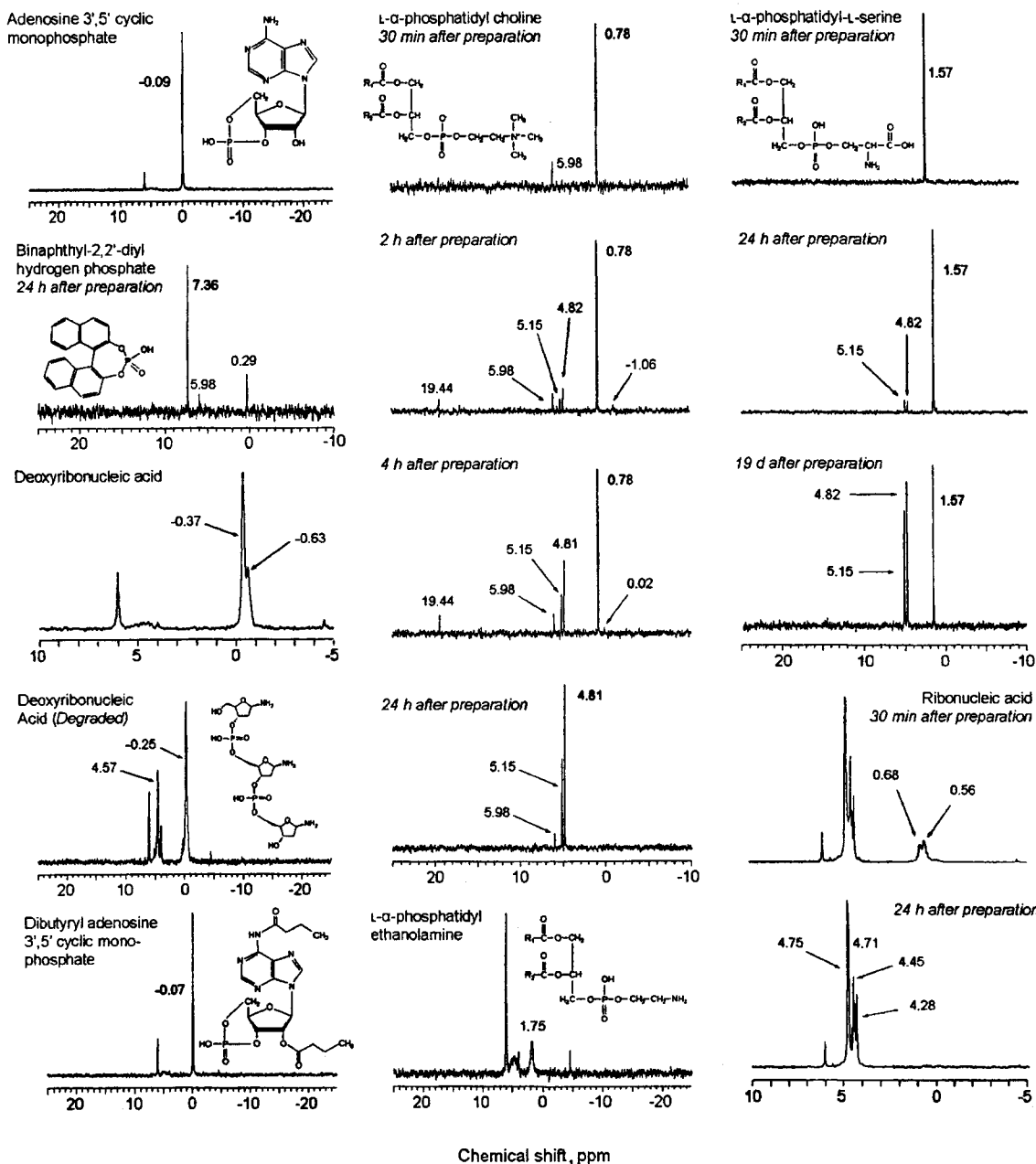


Fig. 3. Solution  $^{31}\text{P}$  NMR spectra of model orthophosphate diesters added to a soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.

clear signals from orthophosphate diesters were present, corresponding to DNA ( $-0.28$  ppm), RNA ( $0.54$  ppm), phosphatidyl serine ( $1.56$  ppm), and phosphatidyl ethanolamine ( $1.66$  ppm). Smaller signals from orthophosphate monoesters appeared between  $4.2$  and  $5.7$  ppm. Storage resulted in the disappearance of signals in the orthophosphate diester region and the appearance of signals in the orthophosphate monoester region corresponding to the degradation products of nucleic acids and phospholipids. The signal corresponding to DNA ( $-0.28$  ppm) was more resistant to degradation than signals corresponding to phospholipids and RNA. Poorly resolved signals between  $0$  and  $1$  ppm probably repre-

sented polynucleotides, as suggested by similar signals in the sample of degraded DNA and RNA (Table 3).

### Effect of Probe Temperature on Solution Phosphorus-31 NMR Spectra

Increasing the probe temperature from  $19$  to  $44^\circ\text{C}$  decreased signal intensity (as a proportion of the initial area at  $19^\circ\text{C}$ ) for all the model compounds analyzed (Fig. 8a). Signals from individual compounds decreased to  $62\%$  of initial spectral area for choline phosphate, to  $76\%$  of original spectral area for *myo*-inositol hexakisphosphate. In the soil extract, this resulted in a smaller proportion of inorganic orthophosphate and a greater

**Table 4. Solution  $^{31}\text{P}$  NMR chemical shifts of model phosphonates in an alkaline soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.**

Compound	Chemical formula	Chemical shift	Line width	Peak area	Orthophosphate shift	Comments
		ppm	Hz	%	ppm	
(R)-(-)-1-aminoethyl phosphonic acid	$\text{C}_2\text{H}_5\text{NO}_3\text{P}$	23.05	11.6	100	6.03	Satellites at 69.2 Hz Satellites at 62.9 Hz No decoupling: sextuplet, $J = 8.7$ Hz
2-aminoethyl phosphonic acid	$\text{C}_2\text{H}_5\text{NO}_3\text{P}$	20.72	9.9	100	6.03	
$\beta$ -naphthyl phenyl phosphonate	$\text{C}_{16}\text{H}_{13}\text{O}_3\text{P}$	13.93	13.4	100	6.05	Signal at 13.94 ppm same height as in fresh solution
1 h after preparation		12.07	13.8	6	6.05	
24 h after preparation		13.94	17.8	94		
		12.06	11.3	45	6.06	
15 d after preparation		13.93	14.2	55		
para-nitrophenyl phenyl phosphonate	$\text{C}_{12}\text{H}_9\text{NO}_3\text{P}$	12.07	10.4	48	6.05	
		13.61	20.8	52		
		0.29	10.4	100	6.05	
Phosphocreatine (N-P bond)	$\text{C}_4\text{H}_{10}\text{N}_3\text{O}_3\text{P}$	0.29	10.4	100	6.05	

proportion of orthophosphate monoesters as probe temperature increased from 18 to 37°C (Fig. 8b). The proportion of the total area assigned to inorganic orthophosphate decreased from 50 to 46%, while the proportion assigned to orthophosphate monoesters increased from 43 to 47%. There were no significant changes in the proportions assigned to orthophosphate diesters or pyrophosphate.

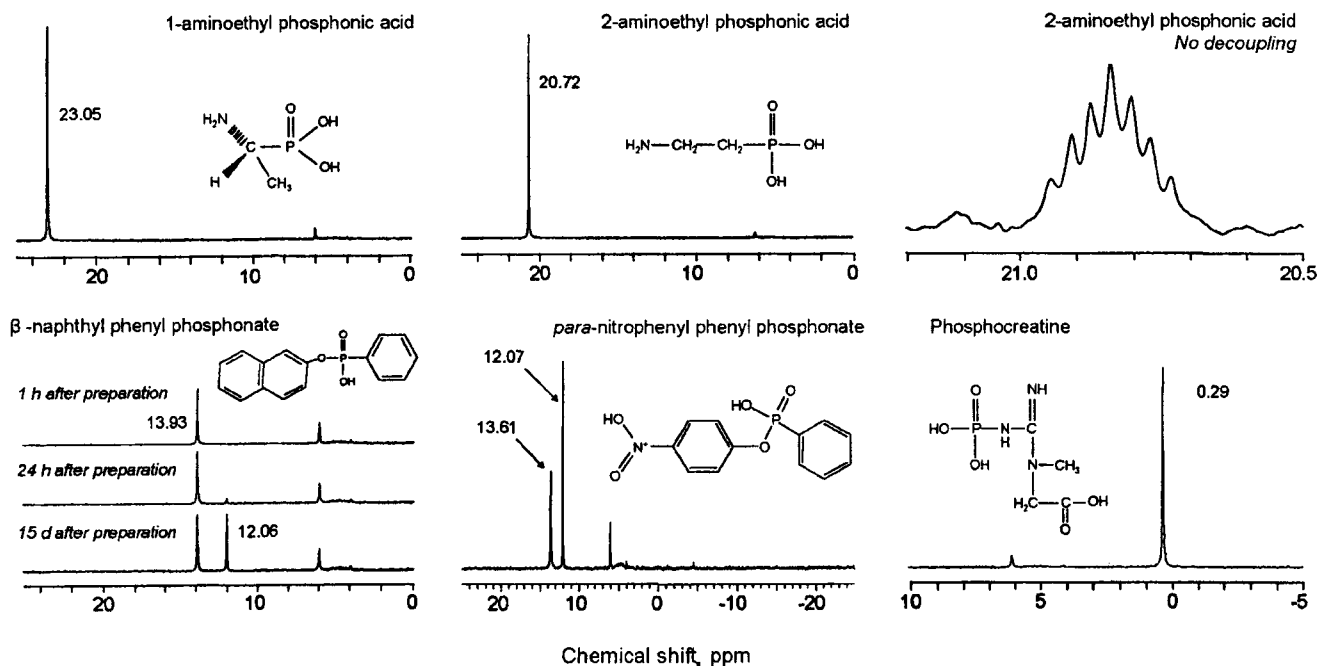
## DISCUSSION

### Chemical Shifts of Model Compounds

The reported chemical shifts of a wide range of P compounds in a standardized soil extract provide a reference for future studies of soil P using solution  $^{31}\text{P}$  NMR spectroscopy. The precise chemical shifts may vary slightly among extracts of different soils and sediments, but the relative positions will remain consistent. In particular, maintaining a solution pH >12 ensures

that the chemical shift of inorganic orthophosphate is reasonably consistent and can be used as a marker to identify other compounds. Importantly, inorganic orthophosphate is distinct from other P compounds at pH >12 except perhaps the most upfield of the four *myo*-inositol hexakisphosphate signals. As solution pH declines, changes in the chemical shift of orthophosphate can interfere with signals from orthophosphate monoesters (Adams, 1990).

Orthophosphate monoesters are the major functional class of organic P in most soils, of which the higher inositol phosphates are usually dominant (Turner et al., 2002b). However, the complex signals from *myo*-inositol hexakisphosphate make it difficult to quantify this compound using solution  $^{31}\text{P}$  NMR spectroscopy. Despite this, it may be possible to discriminate among the range of compounds present in the orthophosphate monoester region using spectral deconvolution software, although this will require the acquisition of well-resolved spectra.

**Fig. 4. Solution  $^{31}\text{P}$  NMR spectra of model phosphonates added to a soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.**



**Table 5.** Solution  $^{31}\text{P}$  NMR chemical shifts of model organic condensed phosphates in an alkaline soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.

Compound	Chemical formula	Chemical shift	Line width	Peak area	Orthophosphate shift	Comments
		ppm	Hz	%	ppm	
Adenosine 5' diphosphate	$\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_{10}\text{P}_2$	-4.67	20.5	28	5.98	$\beta$ doublet: -4.71 ppm, $J = 19.4$ Hz
		-4.75	20.5	26		
		-9.16	23.0	23		$\alpha$ doublet: -9.20 ppm, $J = 17.9$ Hz
		-9.24	23.0	23		
Adenosine 5' triphosphate	$\text{C}_{10}\text{H}_{16}\text{N}_5\text{O}_{13}\text{P}_3$	-4.24	11.2	18	6.00	$\gamma$ doublet: -4.27 ppm, $J = 18.6$ Hz
		-4.31	13.2	21		
		-9.45	9.4	2		doublet: - 9.48 ppm, $J = 17.9$ Hz
		-9.52	9.8			
		-9.65	11.2	14		$\alpha$ doublet: -9.68 ppm, $J = 17.9$ Hz
		-9.72	12.8	17		
		-19.60	12.1	7		$\beta$ triplet: -19.68 ppm, $J = 17.9$ Hz
		-19.68	11.9	13		
-19.76	13.3	8				

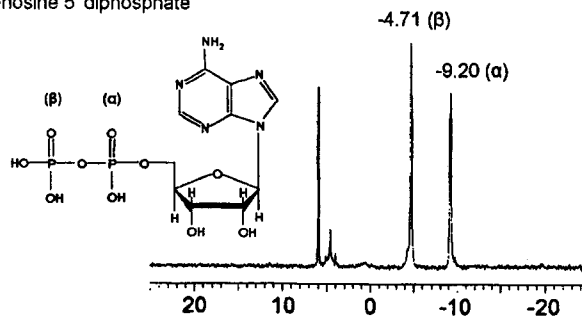
The most readily identified orthophosphate monoesters are glucose-1-phosphate (3.39 ppm) and choline phosphate (4.05 ppm), because of their separation from the main body of signals between 4.3 and 6.0 ppm. Choline phosphate has been previously reported at 4.3 ppm (Adams and Byrne, 1989) and 3.56 ppm (Newman and Tate, 1980), but this is almost certainly accounted for by differences in pH. Adams and Byrne (1989) reported similar chemical shifts to those reported here for glucose-6-phosphate (5.30 ppm) and glycerophosphate (4.78 ppm) in alkaline extracts.

Orthophosphate diesters dominate organic P inputs to the soil from plants and microbes, but are rapidly degraded upon release and typically represent only a small proportion of the soil organic P (Anderson, 1980). Signals from the orthophosphate diesters are commonly grouped together as a single functional class of organic P, but we found reasonable resolution among the various compounds. For example, signals from DNA (-0.37 ppm) and alkali-stable phospholipids (1.5 to 1.8 ppm) were clearly distinguishable. Among the phospholipids, signals from phosphatidyl ethanolamine (1.75 ppm) and phosphatidyl serine (1.56 ppm) were well resolved from phosphatidyl choline (0.78 ppm). The latter may interfere with signals from RNA, although both these compounds were rapidly degraded in alkaline solution (see below). Signals between 0 and 1 ppm were previously assigned to teichoic acids (e.g., Condon et al., 1990), which are present in the cell walls of gram-positive bacteria and are also produced by some actinomycetes (Naumova et al., 2001). However, teichoic acids were recently measured at 1.9 ppm in delipidized extracts of the gram-positive bacteria *Bacillus subtilis* (Makarov et al., 2002), suggesting that they do not contribute greatly to the observed signals in the orthophosphate diester region of alkaline soil extracts. This is possible, because bacterial walls are assembled from P-free teichuronic acid units in P-limited environments (Stewart and Tiessen, 1987).

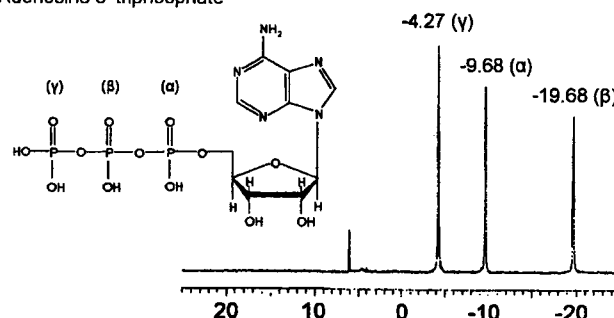
Organic and inorganic polyphosphates both gave similar signals in the negative region of the spectra, but can be differentiated by the presence or absence of an

intermediate signal around -9 ppm from the  $\alpha$  phosphate group of the organic polyphosphates. The chemical shifts reported here for inorganic polyphosphates are similar to literature values for inorganic polyphosphates in alkaline solution, with terminal P groups at approximately -4 ppm and mid-chain P groups giving signals in the -20 ppm region (Adams and Byrne, 1989; Glonek et al., 1975). The high degree of peak splitting in the inorganic polyphosphate spectrum is due to ho-

Adenosine 5' diphosphate



Adenosine 5' triphosphate



Chemical shift, ppm

**Fig. 5.** Solution  $^{31}\text{P}$  NMR spectra of model organic polyphosphates added to a soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.

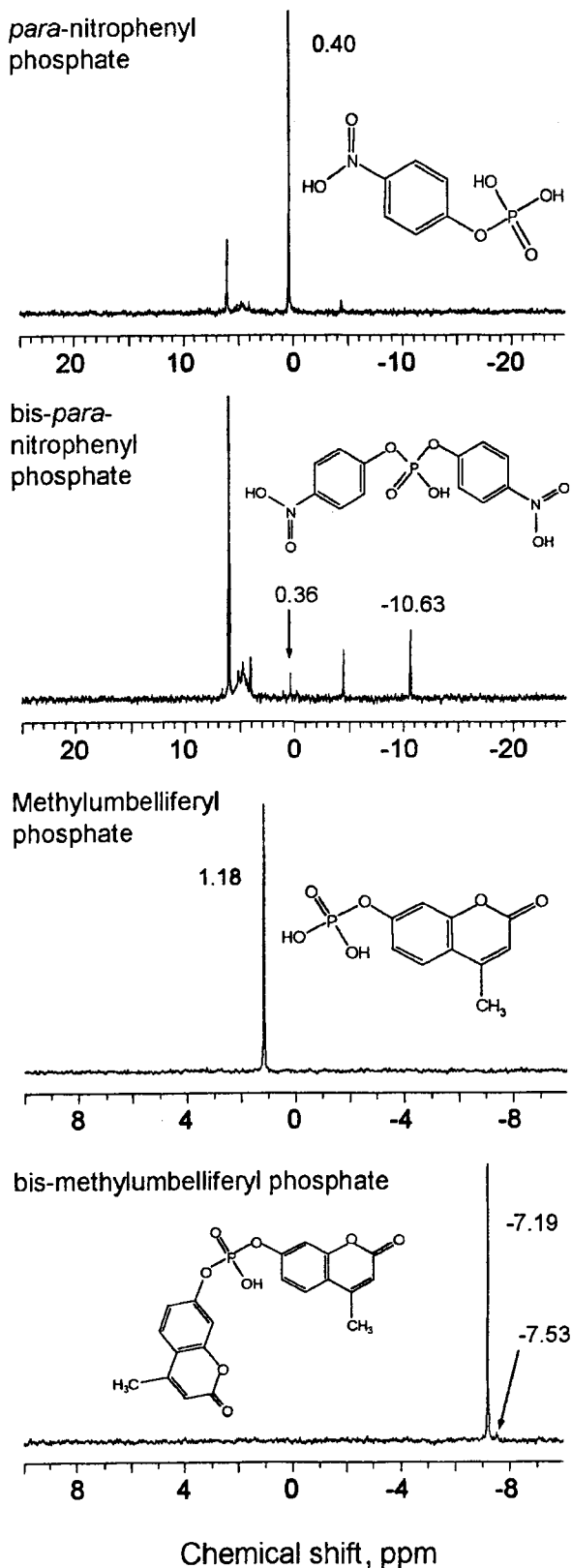


Fig. 6. Solution  $^{31}\text{P}$  NMR spectra of aromatic orthophosphate esters used in the measurement of phosphatase activity added to a soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH. The methylumbelliferyl compounds were dissolved in  $\text{D}_2\text{O}$  and 1 M NaOH only.

monuclear spin-spin coupling induced by the interaction of P nuclei in close proximity.

The spectrum of 2-aminoethylphosphonic acid acquired without proton decoupling highlights the importance of decoupling in the analysis of soil extracts by solution  $^{31}\text{P}$  NMR spectroscopy. The splitting results from heteronuclear spin-spin coupling between  $^1\text{H}$  and  $^{31}\text{P}$  nuclei, which is removed by exciting both nuclei simultaneously. Without proton decoupling, splitting of weak signals from trace amounts of compounds such as phosphonates may result in such compounds being undetected in poorly resolved spectra.

Binaphthyl phosphate is the aromatic diester reported at 7.2 ppm by Bedrock et al. (1994). This appeared slightly downfield of signals from an unidentified compound at 6.9 ppm in soil NaOH–NaF extracts (Amelung et al., 2001), but suggests the presence of an aromatic orthophosphate diester in these samples. However, other aromatic orthophosphate diesters did not give similar signals. Notably, aromatic phosphatase substrates gave widely different signals to most natural phosphate esters, suggesting substantial differences in the chemical environment around the P groups of these structurally different compounds. This has implications for the determination of phosphatase activity, because the enzymes may behave differently toward such aromatic compounds compared with those constituting the bulk of the organic P inputs to the soil. For example, snake venom phosphodiesterase has more than four times the hydrolytic activity toward DNA than bis-*para*-nitrophenyl phosphate (Turner et al., 2002a).

### Degradation of Phosphorus Compounds

Orthophosphate diesters were susceptible to degradation in NaOH–EDTA, but the extent of this varied among the different compounds. For example, the complete hydrolysis of RNA to orthophosphate monoesters within 24 h was in marked contrast to the stability of DNA (Anderson, 1967; Makarov et al., 2002). This at least partly explains the dominance of a signal close to 0 ppm in the orthophosphate diester region of most alkaline soil extracts (e.g., Newman and Tate, 1980; Adams and Byrne, 1989). Of the phospholipids, phosphatidyl choline was the most readily degraded, with almost complete conversion to orthophosphate monoesters within 24 h. Phosphatidyl choline appeared to degrade to glycerophosphate (4.81 ppm) and phosphatidic acid (5.15 ppm), rather than choline phosphate (4.05 ppm). Phosphatidyl serine degraded much more slowly, and phosphatidyl ethanolamine showed no degradation in 24 h. This agrees with the stability of these compounds during 24 h in molar NaOH (Brannon and Sommers, 1985), and suggests that they are responsible for signals between 1 and 2 ppm in the region of alkaline soil extracts. Aromatic orthophosphate diesters also degraded slowly to aromatic orthophosphate monoesters in alkaline solution (Browman and Tabatabai, 1978).

The rapid degradation of some orthophosphate diesters in alkaline solution is an unavoidable limitation on the quantification and characterization of soil organic P,

Table 6. Solution  $^{31}\text{P}$  NMR chemical shifts of aromatic orthophosphate esters used in the measurement of phosphatase activity in an alkaline soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH. The methylumbelliferyl compounds were dissolved in 1 M NaOH only.

Compound	Chemical formula	Chemical shift ppm	Line width Hz	Peak area %	Orthophosphate shift ppm
<i>para</i> -nitrophenyl phosphate (colorimetric monoester)	$\text{C}_6\text{H}_4\text{NO}_6\text{P}$	0.40	12.5	100	6.08
bis <i>para</i> -nitrophenyl phosphate (colorimetric diester)	$\text{C}_{12}\text{H}_8\text{N}_2\text{O}_8\text{P}_2$	0.35 -10.63	8.9 13.4	20 80	6.02
methylumbelliferyl phosphate (fluorimetric monoester)	$\text{C}_{10}\text{H}_8\text{O}_6\text{P}$	1.18	7.4	100	
bis-methylumbelliferyl phosphate (fluorimetric diester)	$\text{C}_{20}\text{H}_{16}\text{O}_8\text{P}_2$	-7.19 -7.53	6.3 17.6	95 5	

because alkaline solutions are required to extract organic P from most soils (Anderson, 1980). Strong-acid extraction techniques can extract large proportions of organic P from some soils (Bowman, 1989), but are unsuitable for subsequent  $^{31}\text{P}$  NMR analysis without additional treatment. Therefore, the degradation of some compounds must be accepted as an artifact of soil organic P analysis by current procedures. Given the rapid nature of the degradation of RNA and phosphatidyl choline, it seems probable that most degradation will

occur during extraction and lyophilization. Therefore, the possibilities to reduce this are limited, although the rehydration of freeze-dried extracts immediately before NMR analysis is advisable. It is possible that the nature of orthophosphate diesters in the soil (such as in complexes with humic material), may limit their degradation in alkaline solution, but this seems unlikely, at least for RNA (Makarov et al., 2002). Many orthophosphate diesters are derived from soil microbes, which can represent a considerable proportion of the soil organic P

Table 7. Solution  $^{31}\text{P}$  NMR chemical shifts of P compounds in bacteria cultured from an upland blanket peat from northern England. Bacteria were added to an alkaline soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.

Time after sample preparation	Chemical shift ppm	Line width Hz	Peak area %	Comments
30 min	-1.07-0.00		20	Main signal -0.28 ppm (6%)
	0.00-1.00		11	Main signal 0.54 ppm (6%)
	1.00-1.47		4	
	1.56	15.6	6	
	1.66	12.7	8	
	1.77-2.26		2	
	4.22-5.39		11	
	5.88	14.2	35	
	18.95	33.8	2	
24 h	-0.54-0.00		16	Main signal -0.27 ppm (7%)
	0.00-1.00		1	
	1.00-1.48		4	
	1.57	22.0	2	
	1.67	14.3	5	
	1.78	19.2	3	
	4.23-5.00		25	Main signals 4.67, 4.50 ppm (7% each)
	5.00-5.57		7	
	5.89	14.2	37	
	21.70	33.8	1	
9 d	-0.70-0.00		13	Main signal -0.26 ppm (5%)
	0.00-1.40		4	
	1.48	14.2	3	
	1.62	15.2		
	1.67	12.8		
	1.76	10.4		
	3.21	13.2	1	
	3.70-5.00		31	Main signals 4.73 (10%), 4.66 (4%), 4.42 (3%) ppm
	5.00-5.58		13	Main signals 5.07 (7%), 5.00 ppm (4%)
	5.88	12.0	36	
23 d	-0.70-0.00		13	Main signal -0.26 ppm (7%)
	0.00-1.40		3	
	1.48	14.2	2	
	1.62	15.2		
	1.67	12.8		
	1.76	10.4		
	3.21	13.2	1	
	3.70-5.00		29	Main signals 4.73 (12%), 4.66 (3%), 4.61 (3%), 4.42 (3%) ppm
	5.00-5.58		15	Main signals 5.07 (8%), 5.00 ppm (4%)
	5.88	12.0	38	

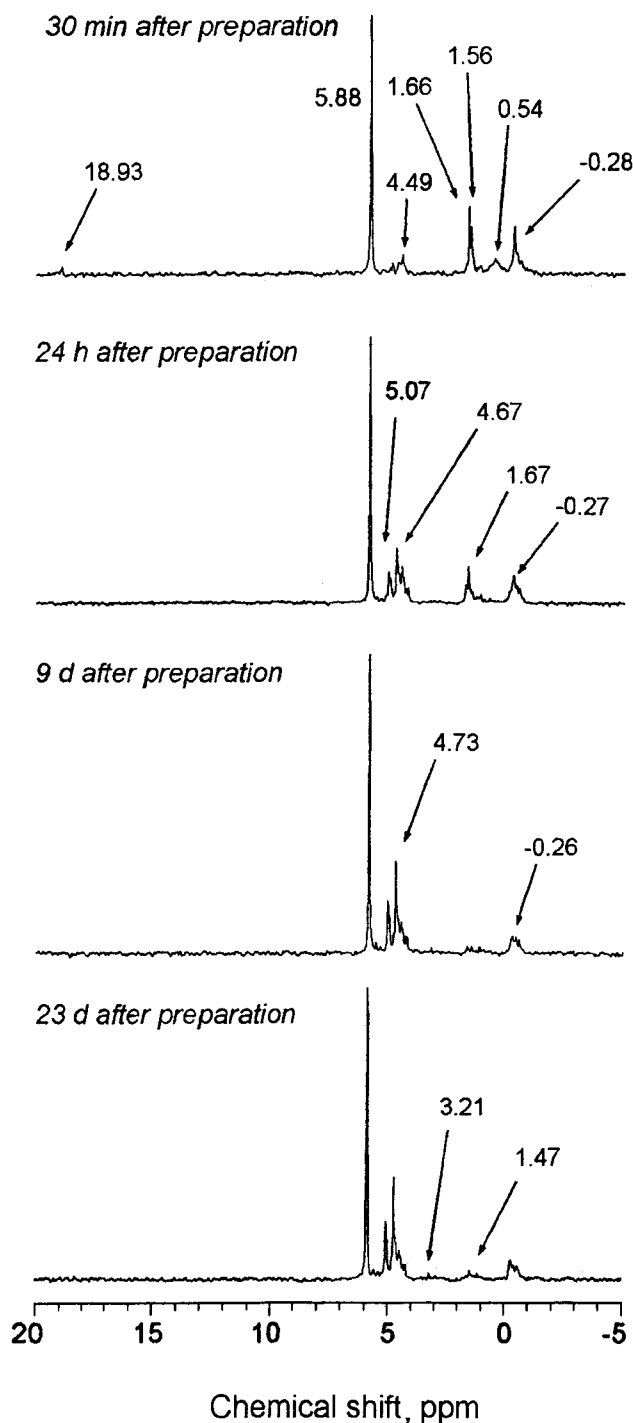


Fig. 7. Degradation of organic P compounds in bacteria cultured from an upland blanket peat between 0 and 23 d after preparation. Bacteria were added to an alkaline soil extract (0.25 M NaOH and 0.05 M EDTA) that had been lyophilized and redissolved in  $\text{D}_2\text{O}$  and 1 M NaOH.

(Turner et al., 2001b), so the greatest errors may occur in soils high in microbial biomass.

Based on the chemical shifts of orthophosphate diesters, and their relative degradation in alkaline solution, we suggest that the orthophosphate diester region of solution  $^{31}\text{P}$  NMR spectra of alkaline soil extracts can be

reclassified into three groups of compounds: (i) DNA between -1.0 and 0 ppm, (ii) alkali-labile diesters (RNA and phosphatidyl choline) between 0 and 1.0 ppm, and (iii) microbial phospholipids between 1.5 and 1.8 ppm. In addition, teichoic acids may also be identified at approximately 1.9 ppm in extracts of some soils (Makarov et al., 2002). This refines peak assignments and will enhance the information obtained from solution  $^{31}\text{P}$  NMR spectroscopy of alkaline soil extracts. The assignments were confirmed by the presence and degradation of orthophosphate diesters in soil bacteria corresponding to RNA (0.54 ppm), DNA (-0.28 ppm), phosphatidyl serine (1.56 ppm), and phosphatidyl ethanolamine (1.66 ppm).

Organic and inorganic polyphosphates were stable in NaOH-EDTA, suggesting that their absence in alkaline soil extracts is not explained by degradation during analytical procedures. Long-chain polyphosphates degrade to shorter chain lengths in the presence of metal ions in sediment extracts (Hupfer and Gächter, 1995), but degradation is slowed when EDTA is included in the initial extract. This is probably due to the complexation of free metals, and confirms the suitability of NaOH-EDTA as an extractant for soil polyphosphates (Cade-Menun and Preston, 1996).

Two signals are sometimes detected in the 20 ppm region of soil extracts, probably representing a compound such as 2-aminoethyl phosphonic acid around 21 ppm and a higher molecular weight phosphonolipid (e.g., phosphonylethanolamine) around 19 ppm (Newman and Tate, 1980). Degradation of the latter to an alkyl phosphonate has been reported in alkaline solution (Newman and Tate, 1980; Cade-Menun et al., 2002). We detected no degradation of aminoethyl phosphonic acids in NaOH-EDTA, but we did not include a phosphonolipid in our experiments.

### Effect of Probe Temperature on Phosphorus-31 NMR Spectra

Small changes in chemical shifts of signals from organic P compounds in response to temperature changes are widely reported and, at least for orthophosphate diesters, appear to be linked to conformational changes (Gorenstein, 1984). However, increases in temperature can also influence spin-lattice relaxation times ( $T_1$  times), which may explain the decreases in signal intensity with increases in temperature (Crouse et al., 2000). For a  $^{31}\text{P}$  NMR experiment to be quantitative, the pulse delay time must be sufficient to allow nuclei to fully relax following excitation (Cade-Menun et al., 2002). If nuclei fail to fully relax (pulse delay  $< T_1$ ), which may occur as temperature increases, then signal intensities will be smaller than for fully relaxing nuclei. Changes in the relative intensities of orthophosphate monoesters and inorganic orthophosphate add further complication. Therefore, we suggest that solution  $^{31}\text{P}$  NMR experiments should be conducted at a standardized temperature close to 20°C, as suggested elsewhere (Crouse et al., 2000; Cade-Menun et al., 2002). This will ensure comparability among studies, reduce the number of scans required to obtain acceptable signals, and minimize temperature-associated degradation of compounds.

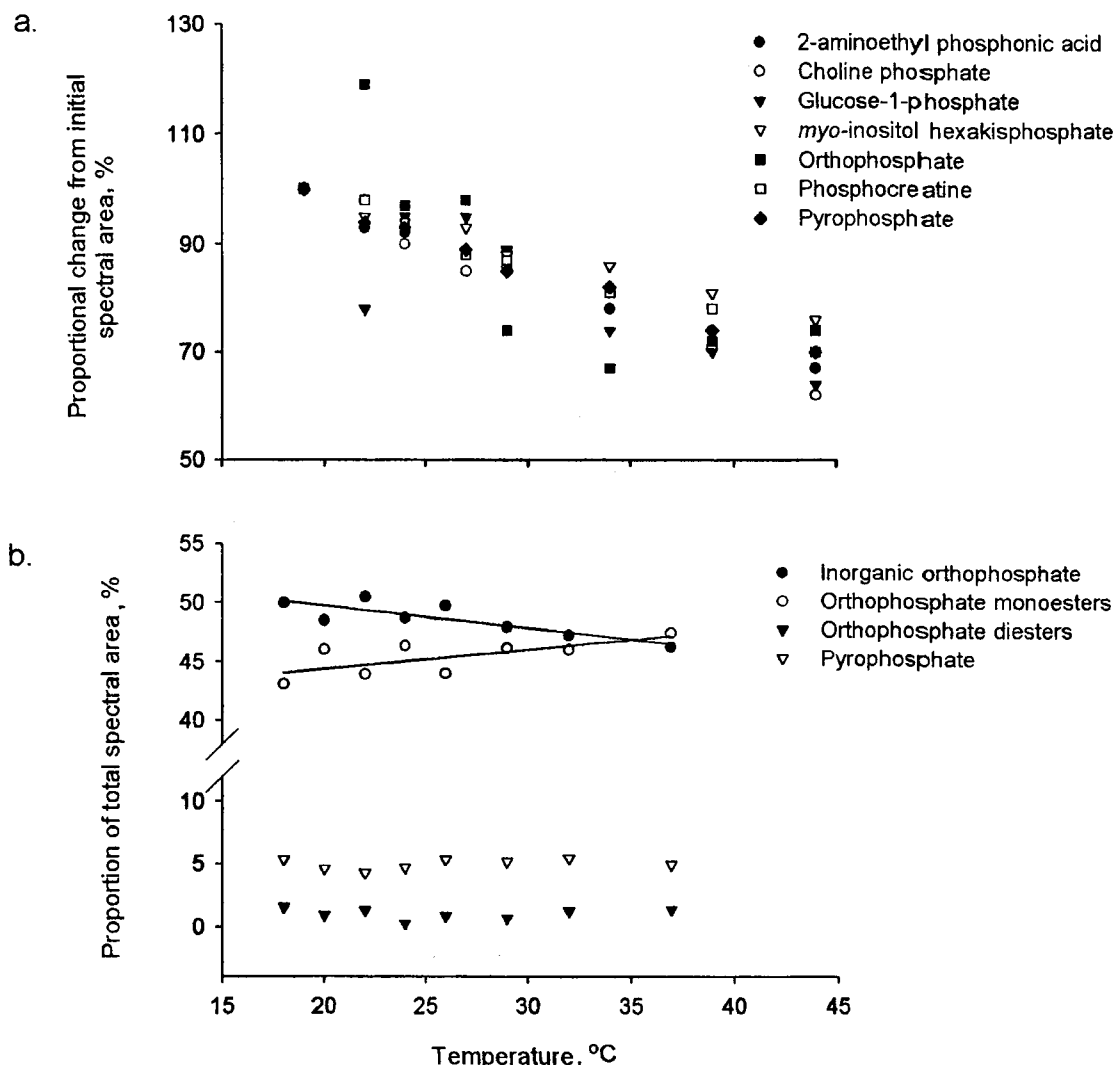


Fig. 8. The effect of probe temperature on (a) spectral areas of model P compounds in a synthetic mixture of 1 M NaOH (proportion of the initial spectral area at 19°C) and (b) proportion of the spectra assigned to the various functional P classes in a 0.25 M NaOH and 0.05 M EDTA soil extract (%). The regression lines on Fig. 3b describe the following models: [Proportion of orthophosphate (%)] =  $53.49 \pm 1.36 - 0.188 \pm 0.051[\text{probe temperature } (^{\circ}\text{C})]$ ,  $R^2 = 0.69$ ,  $F = 13.6$ ,  $P = 0.010$ ; [Proportion of orthophosphate monoesters (%)] =  $41.08 \pm 1.83 + 0.165 \pm 0.069[\text{probe temperature } (^{\circ}\text{C})]$ ,  $R^2 = 0.49$ ,  $F = 5.78$ ,  $P = 0.053$ .

## CONCLUSIONS

A wide range of inorganic and organic P compounds can be identified in soil NaOH-EDTA extracts by solution <sup>31</sup>P NMR spectroscopy. In particular, signals from nucleic acids (DNA and RNA) and microbial phospholipids (phosphatidyl ethanolamine and phosphatidylserine) can be differentiated, while inorganic and organic polyphosphates can be differentiated by the presence of a signal in the -9-ppm region from the  $\alpha$  phosphate group of ADP and ATP. Some orthophosphate diesters degrade in the alkaline conditions used to extract and analyze soil organic P, notably RNA and phosphatidylcholine, which may limit the accurate determination of soil P composition by solution <sup>31</sup>P NMR spectroscopy. However, other compounds, including DNA and microbial phospholipids, are more stable, at least in the time frame of <sup>31</sup>P NMR experiments. Relative signal intensities from inorganic orthophosphate and orthophosphate

monoesters vary with probe temperature, so a standard operating temperature of 20°C is recommended for solution <sup>31</sup>P NMR analysis.

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